Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Photoinduced toxicity mechanisms of AgNPs to microalgae *Chlorella pyrenoidosa* in the presence of hematite nanoparticles: insights from transcriptomics, metabolomics and the photochemical index

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Pages: 23
Materials and methods: S2-S8
Figures: S9-S20
Tables: S21-S23

S1 Percentage of growth rate inhibition assay

The optical density of *Chlorella pyrenoidesa* (*C. pyrenoidesa*) at wavelength of 683 nm (OD_{683}) was measured at 0 h and 96 h by a ultraviolet–visible spectrophotometer. The average specific growth rate of *C. pyrenoidesa* was calculated using the following equation [1]. Then, according to the average specific growth rate, the percentage of growth rate inhibition in each parallel treatment sample was calculated by equation [2]:

$$\mu_{i-j} = \frac{lnX_j - lnX_i}{t_j - t_i} (day^{-1}) \quad \text{-----[1]}$$

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad \text{-----[2]}$$

where:

μ_{i-j}: average specific growth rate from time i to time j; X_i: value of OD_{683} at time i; X_j: value of OD_{683} at time j; $\%I_r$: percentage of average specific growth rate inhibition; μ_C: mean average specific growth rate value (μ) in the control group; μ_T: average specific growth rate in the treatment group replicates.

S2 Determination of pigments

C. pyrenoides (10 mL) was centrifuged at 9000 rpm for 10 min, and then the residues were suspended in 90% ethyl alcohol (10 mL). The obtained samples were kept in the dark at 4 ℃ for 24 h before centrifugal treatment at 12000 rpm for 10 min. The optical density (OD) of the supernatants was measured at 469 nm, 644 nm, 665 nm and 750 nm to determine the chlorophyll a, chlorophyll b and carotenoid contents. The chlorophyll a, chlorophyll b and carotenoid contents were calculated using the following formulas [3], [4] and [5], respectively.

Chlorophyll a (mg/L) = $C_a = 13.95 \times (A_{665}-A_{750})-6.88 \times (A_{644}-A_{750})$ -----[3]

Chlorophyll b (mg/L) = $C_b = 24.96 \times (A_{644}-A_{750})-7.32 \times (A_{665}-A_{750})$ -----[4]

Carotenoid (mg/L) = $[1000 \times (A_{469}-A_{750})-2.05C_a-114.8C_b]/248$ -----[5]

S3 Determination of membrane damage

Fluorescein diacetate (FDA) was used as a fluorescent probe to measure cell membrane permeability. After 96 h of exposure, algal cells were collected and centrifuged at 9000 rpm at 4 ℃ for 10 min. After removing the supernatant, the algal cells were collected and washed with BG11 medium three times. The samples were then incubated with 10 μM FDA in the dark at 37 ℃ for 30 min and rinsed with BG11 medium three times. Finally, the fluorescence intensity was measured by a fluorescence spectrophotometer (F 2700, Japan).

S4 Determination of ROS

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect ROS. DCFH-DA is hydrolyzed by intracellular esterase to produce DCFH. In the presence of ROS, DCFH is oxidized to the fluorescent substance DCF. After 96 h of exposure, algal cells were collected and centrifuged at 9000 rpm at 4 ℃ for 10 min. After removing the supernatant, the algal cells were collected and washed with BG11 medium three times. The samples were then incubated with 10 μM FDA in the dark at 37 ℃ for 30 min and rinsed with BG11 medium three times. Finally, the level of ROS was measured by a microplate reader (Spectra Max Plus384, USA).

S5 Transcriptomic Analysis

C. pyrenoidesa cells in the CK, AgNPs, HemNPs, MX1 and MX2 groups were separated from the supernatants by centrifugation (3000 rpm, 15 min). Total RNA was extracted from the *C. pyrenoidesa* cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Then, RNA-seq transcriptome libraries were constructed and sequenced with the assistance of Majorbio Biopharm Technology Co., Ltd. mRNA can be isolated from total RNA by A-T base pairing between magnetic beads with oligo (dT) and poly-(A) attachments. The messenger RNA was randomly broken by adding fragmenting buffer, and small fragments were isolated by magnetic-bead screening. Using the mRNA as the template, one-stranded cDNA was synthesized by inversion, and then two-stranded cDNA synthesis was carried out to form a stable double-stranded structure. End-repair mixture was added to provide the double-stranded cDNA with a flat end for sequencing on the Illumina platform. The sequences were annotated in seven public databases (NR, NT, PFAM, SWISS-PROT, KOG, KEGG and GO) for subsequent functional interpretation and metabolic pathway analysis. FPKM (fragments per kilobases per million reads) values were used to normalize the transcript abundances. Genes with fold changes > 2 and p values < 0.05 were identified as significantly differentially expressed genes (DEGs) between two groups. The DEGs (p-adjust < 0.05 & |log2 FC| >= 1) were analyzed using the differential analysis software DESeq2 (version 1.38.0).

S6 Metabolomic Analysis

After 96 h of exposure, different groups of *C. pyrenoidesa* cells were collected by centrifugation for metabolomic analysis. The cells were then washed three times with PBS buffer. Subsequently, the washed cell samples were stored at −80 °C until further analysis. Metabolomic analysis was performed by liquid chromatography–mass spectrometry (LC–MS, ExionLC AD System, ABSCIEX-Triple TOF 5600, AB SCIEX, USA). The specific steps were as follows. Each algal sample (50 mg) was mixed with 20 μL of internal standards (L-2-chloro-phenylalanine dissolved in acetonitrile, 0.3 mg/mL) and added to 400 μL methanol/water (4:1, v/v). Subsequently, the mixture was by a high-throughput tissue-crushing apparatus (Wonbio-96c, Shanghai Wanbai Biotechnology Co., Ltd., China) at -20 ℃ and 50 Hz for 6 min and vortexed. The obtained mixtures were treated by ultrasonic solvent extraction (40 kHz) at 5 ℃ for 30 min and then held at -20 ℃ for 30 min. The supernatants containing extracted metabolites were obtained by centrifugation at 13000 g and 4 ℃ for 15 min prior to drying by nitrogen flushing. The collected mixtures were reconstituted in acetonitrile/water (1:1, v/v) and analyzed by liquid chromatography–mass spectrometry (LC–MS, ExionLC AD System, ABSCIEX-Triple TOF 5600, AB SCIEX). Samples were injected into BEH C18 columns (100 mm × 2.1 mm i.d., 1.7 μm; Waters, Milford, USA) using a UPLC-TripleTOF system with mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile/isopropanol, 1:1, 0.1% formic acid). The flow rate was 0.40 mL/min, the injection volume was 10 μL, and the column temperature was 40 ℃.

Mass spectrometry spectra of the samples were collected with positive (ESI+) and negative (ESI-) ion scanning modes and the ion spray voltage. The original LC – MS data were normalized and transformed, and then unsupervised principal component analysis (PCA) and orthogonal projection latent structure discriminant analysis (OPLS-DA) were carried out. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and the human metabolome database (HMDB) were used for metabolic pathway analysis and compound classification. Metabolites with significant differences between two groups were screened by t tests. Metabolites with $p < 0.05$ and VIP > 1 were considered significantly different between two groups.

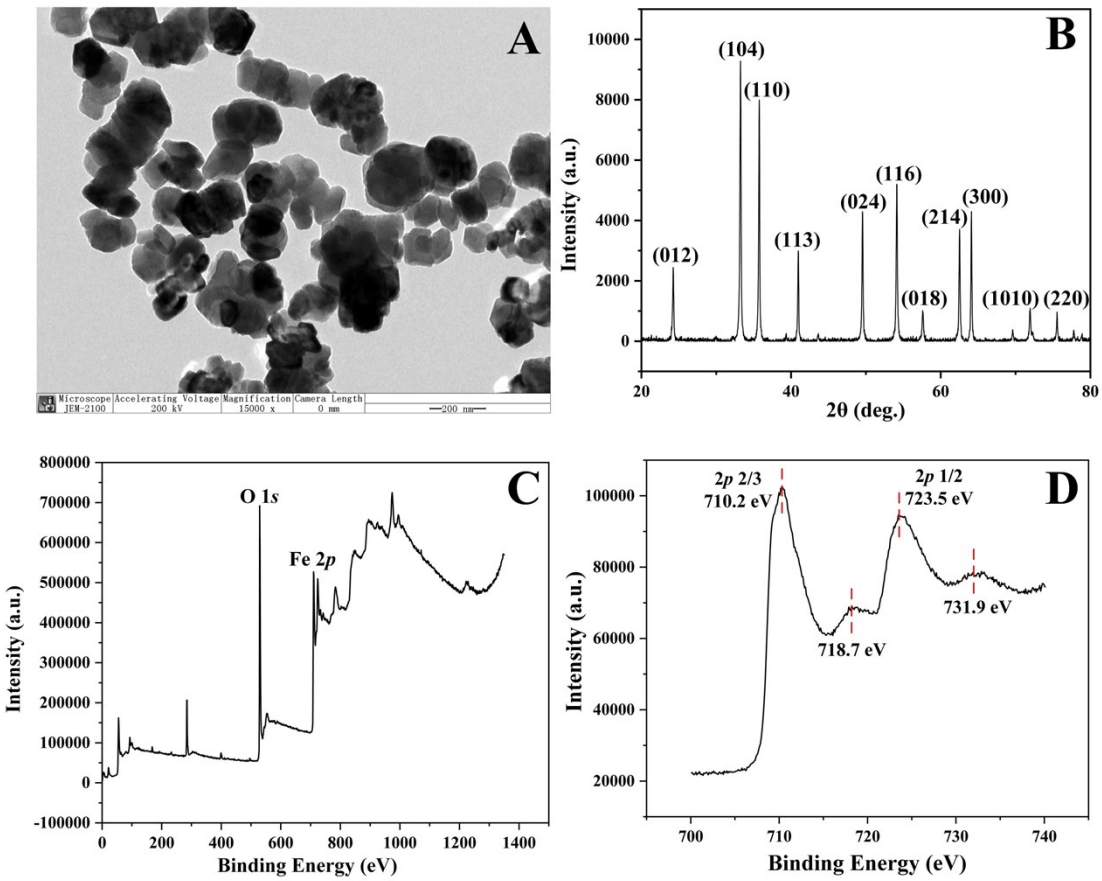

Fig. S1 (A) TEM of HemNPs. (B) X-Ray Powder Diffraction of HemNPs. (C-D) X ray photoelectron spectrum of HemNPs: General layout (C); Fe 2*p* (D).

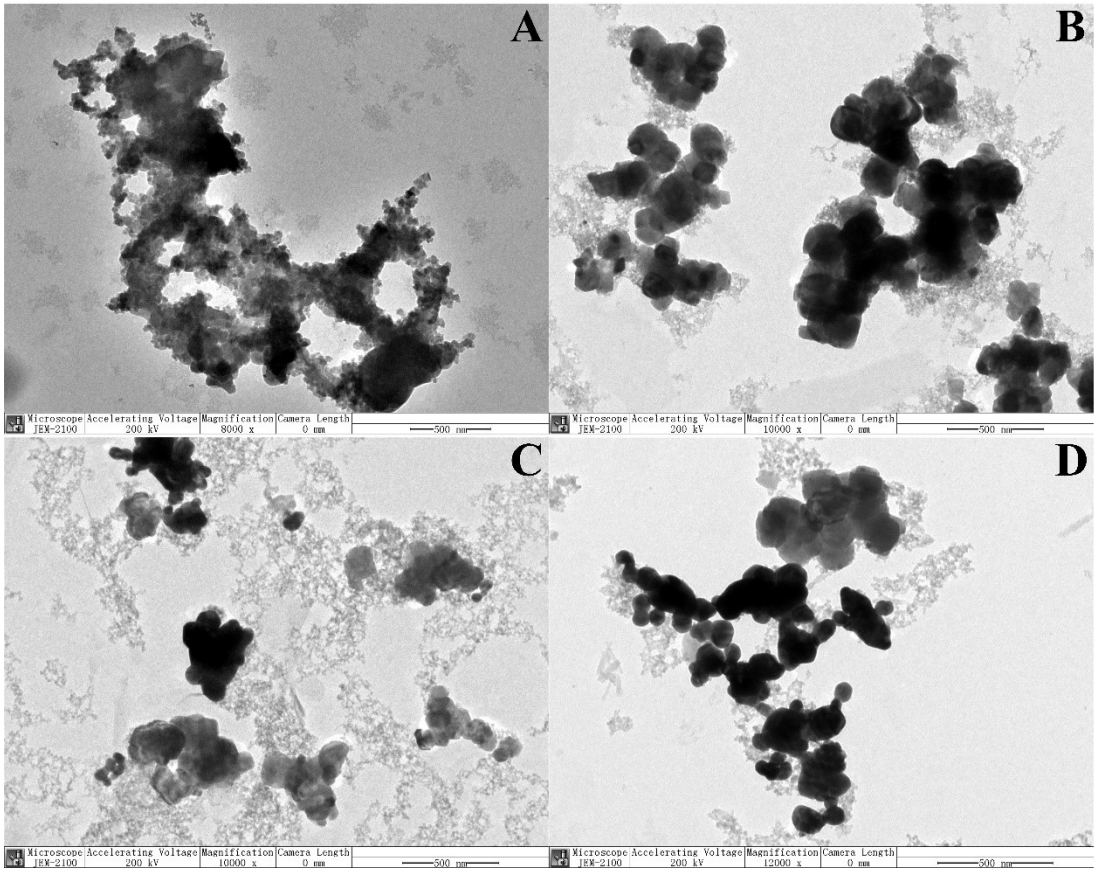

Fig. S2 Transmission electron microscopy (TEM) images of (A) AgNPs, (B) HemNPs, (C) MX1 and (D) MX2 in BG11 medium.

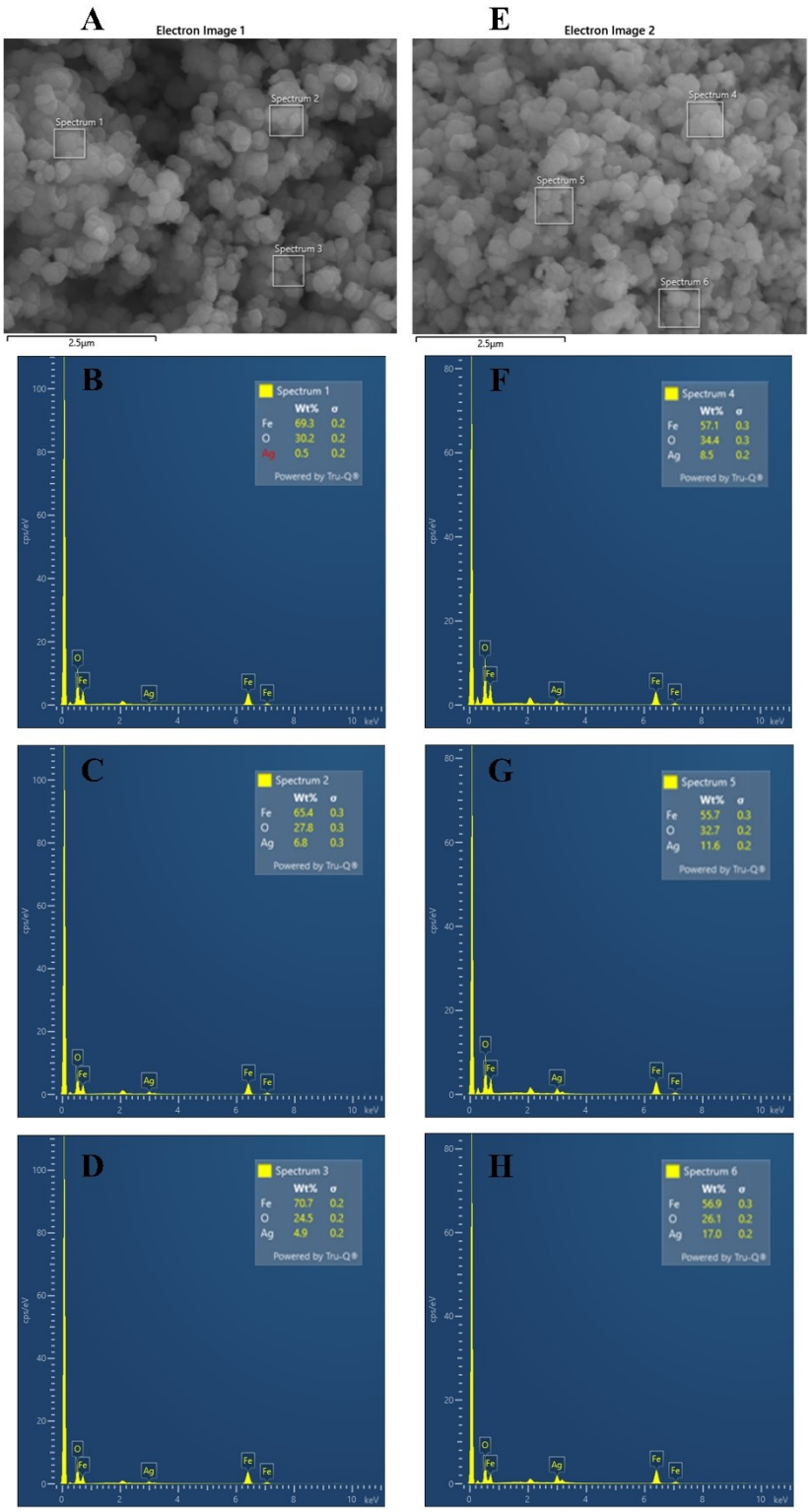

Fig. S3 SEM micrograph and corresponding EDS spectra for the composites of AgNPs and HemNPs. (A-D) AgNPs and HemNPs were mixed for two days. (E-H) AgNPs and HemNPs were mixed for six days.

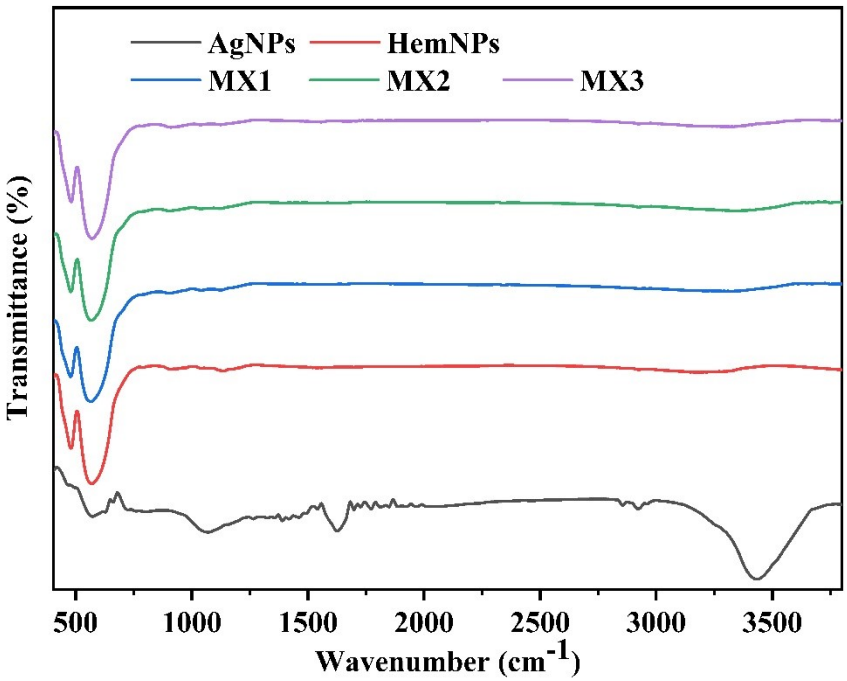

Fig. S4 Infrared spectra of AgNPs, HemNPs and their composites. MX1-3: AgNPs and HemNPs were mixed for zero/two/six days.

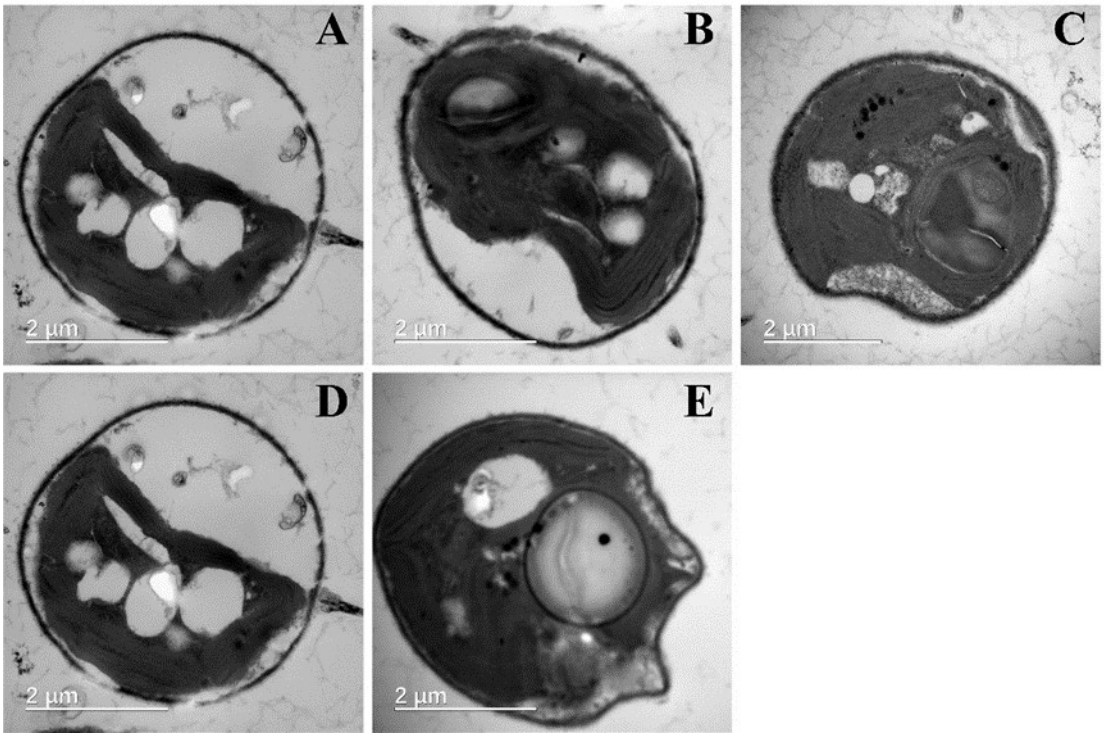

Fig. S5 STEM images of *C. pyrenoidesa*: (A) CK; (B) AgNPs; (C) HemNPs; (D)MX1; (E)MX2.

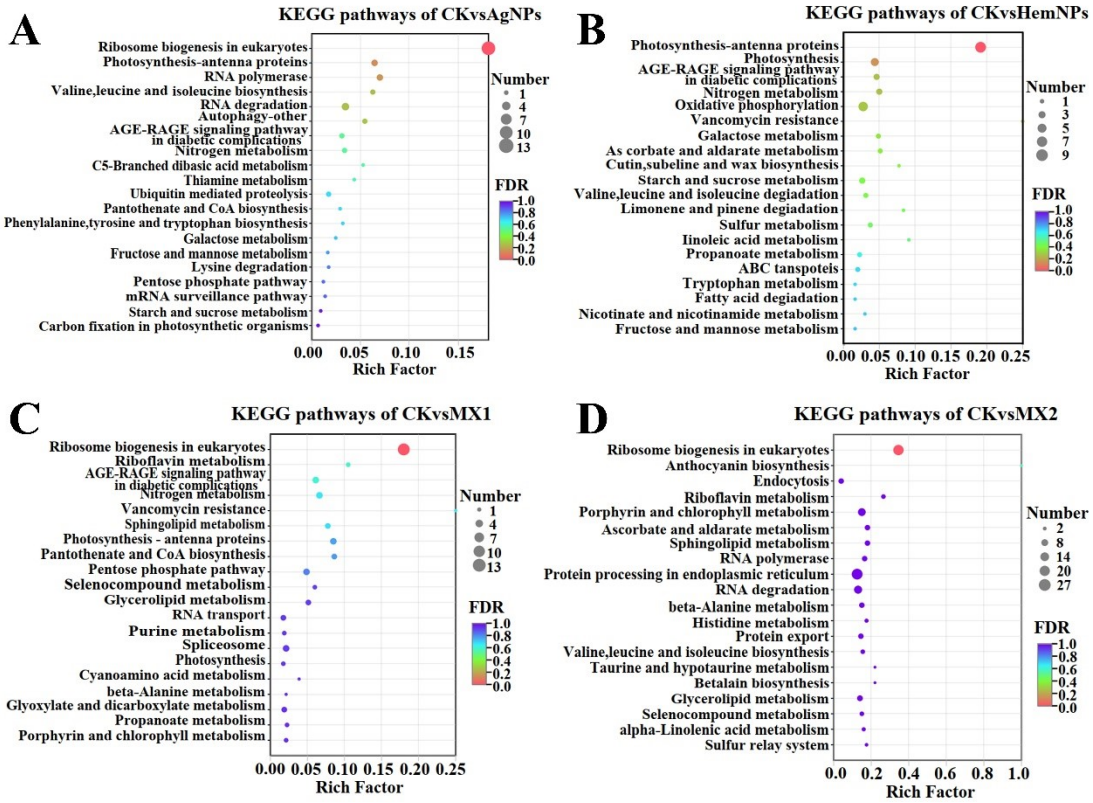

Fig. S6 KEGG functional classification of differentially expressed genes (DEGs).

(A) CK vs AgNPs. (B) CK vs HemNPs. (C) CK vs MX1. (D) CK vs MX2.

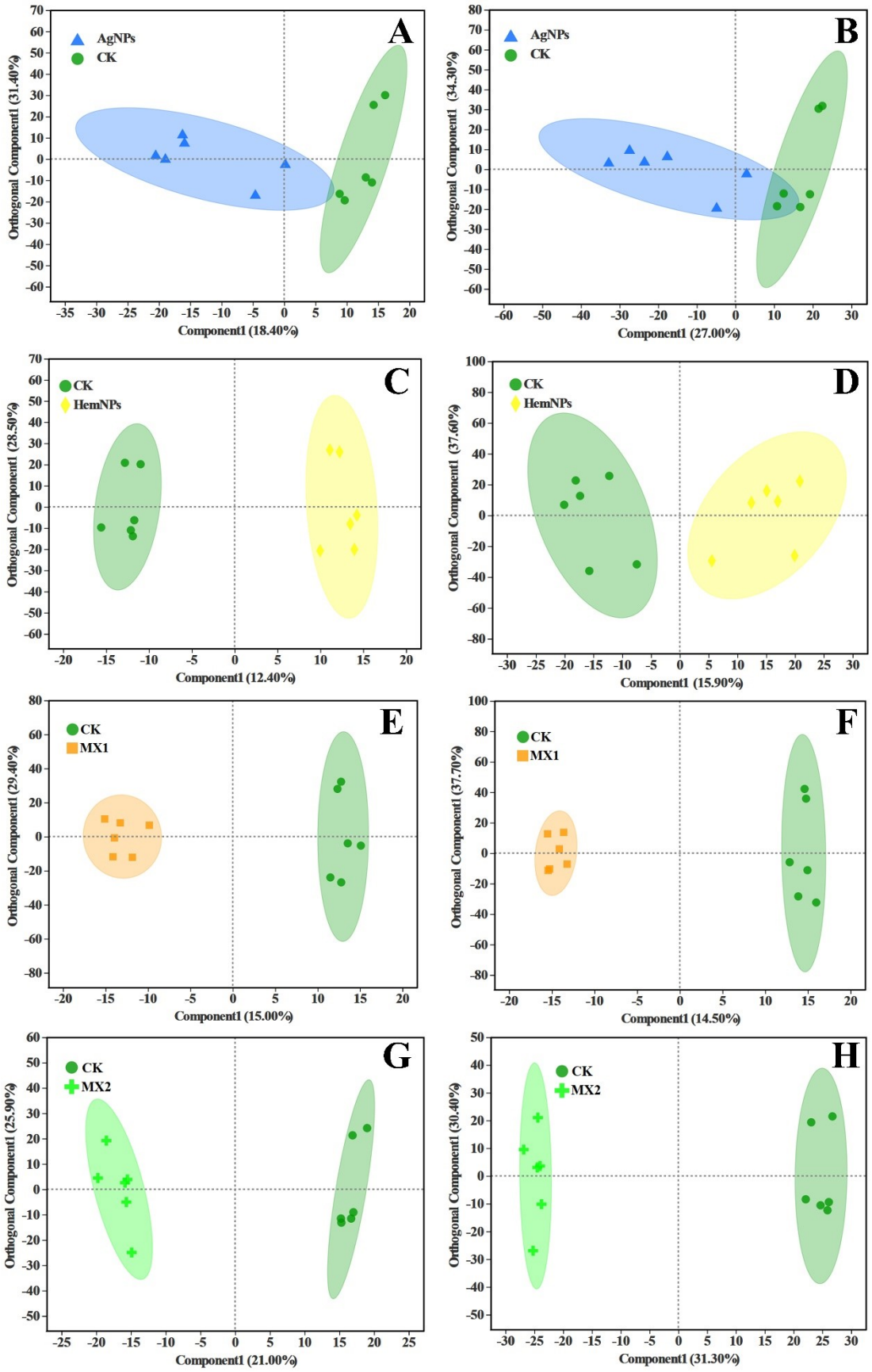

Fig. S7 OPLS-DA of DMs (left: positive mode; right: negative mode).

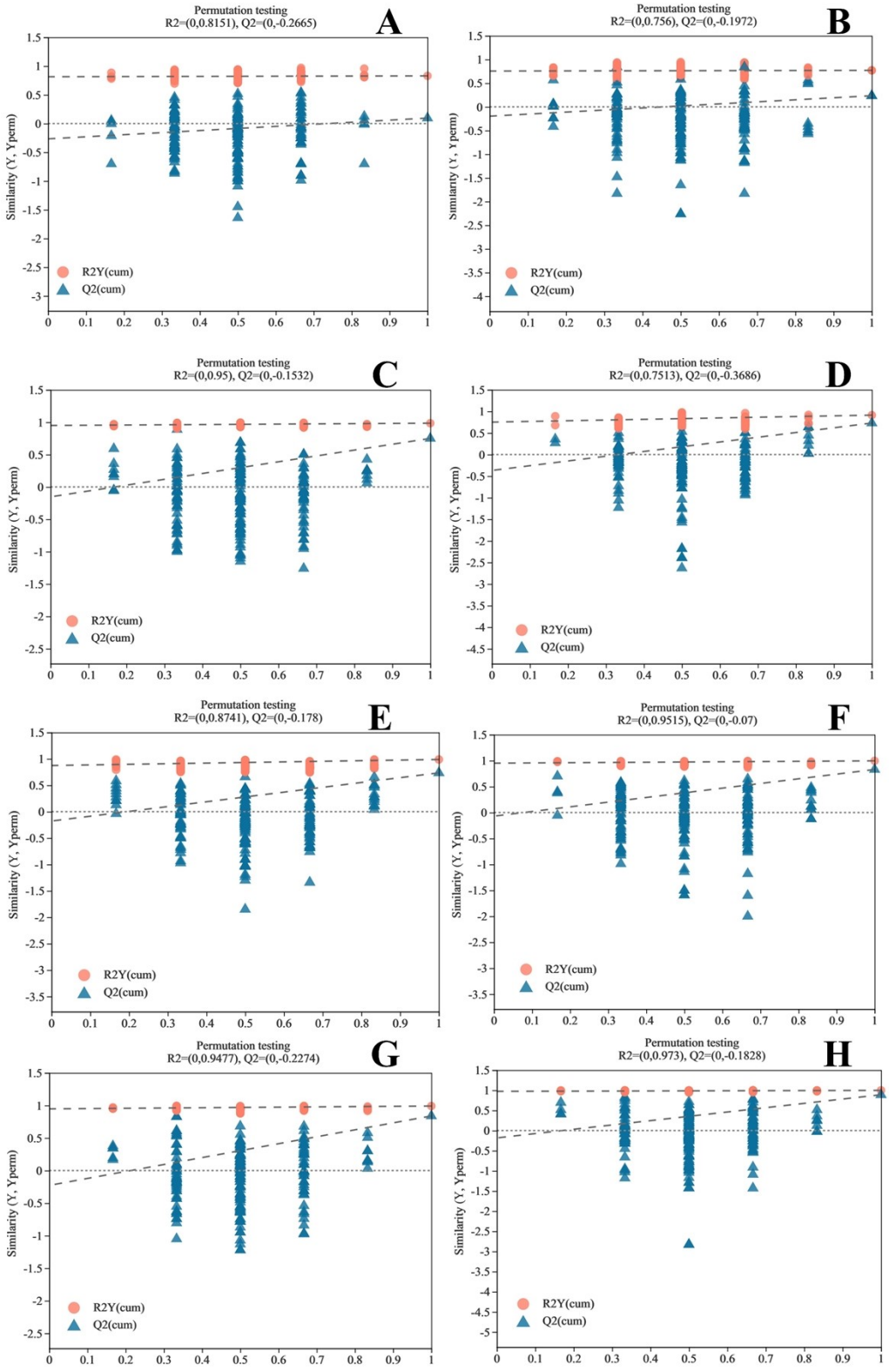

Fig. S8 Validation plots of OPLS-DA obtained from 200 tests. A-B: AgNPs vs CK, C-D: HemNPs vs CK, E-F: MX1 vs CK, E-F: MX2 vs CK (left: positive mode; right: negative mode).

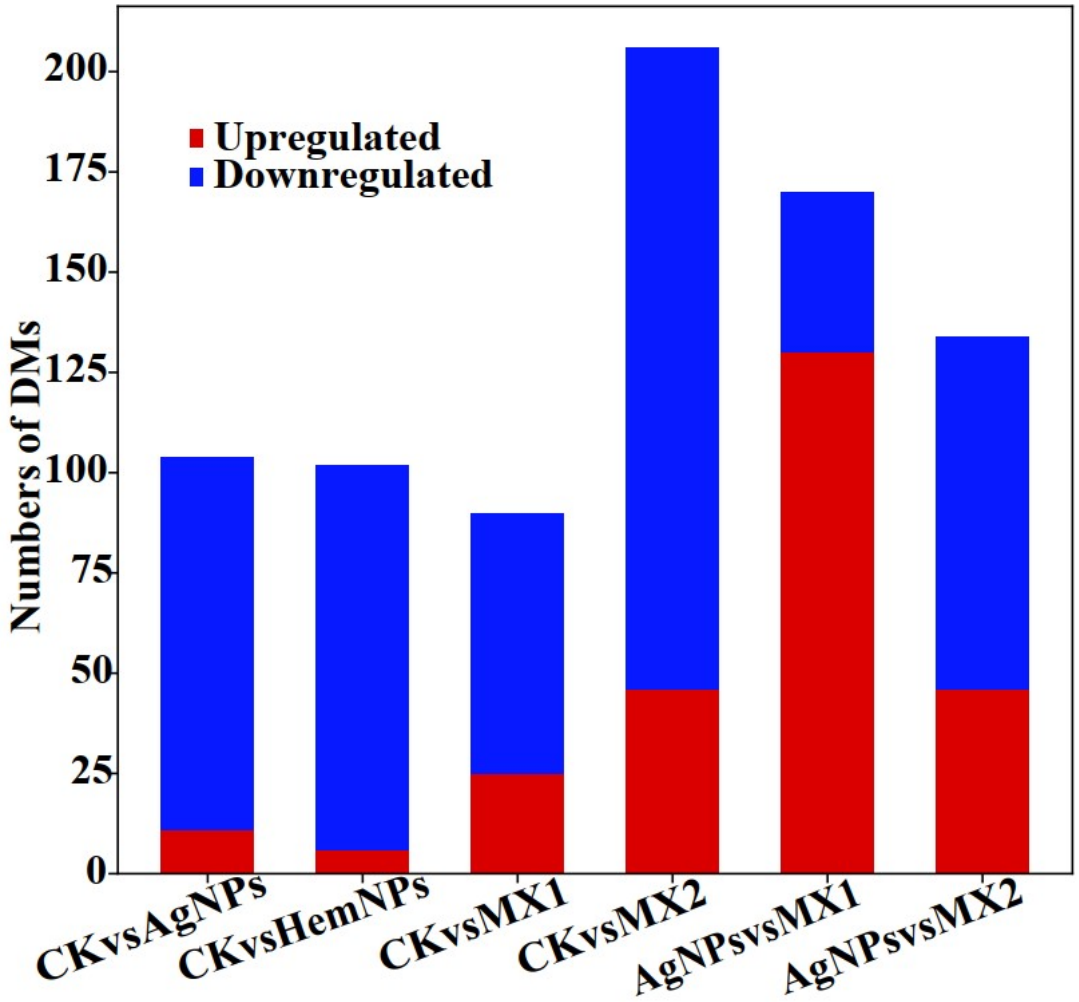

Fig. S9 Histogram of the number of DMs in the single and combined exposure of AgNPs and HemNPs compared with the control.

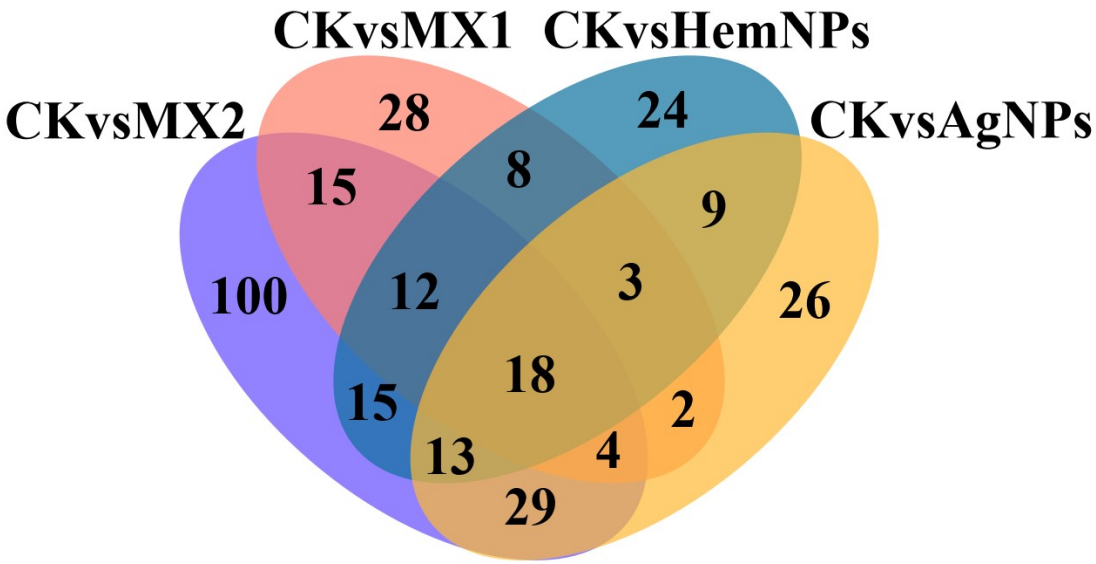

Fig. S10 Venn diagram showing the number of DMs.

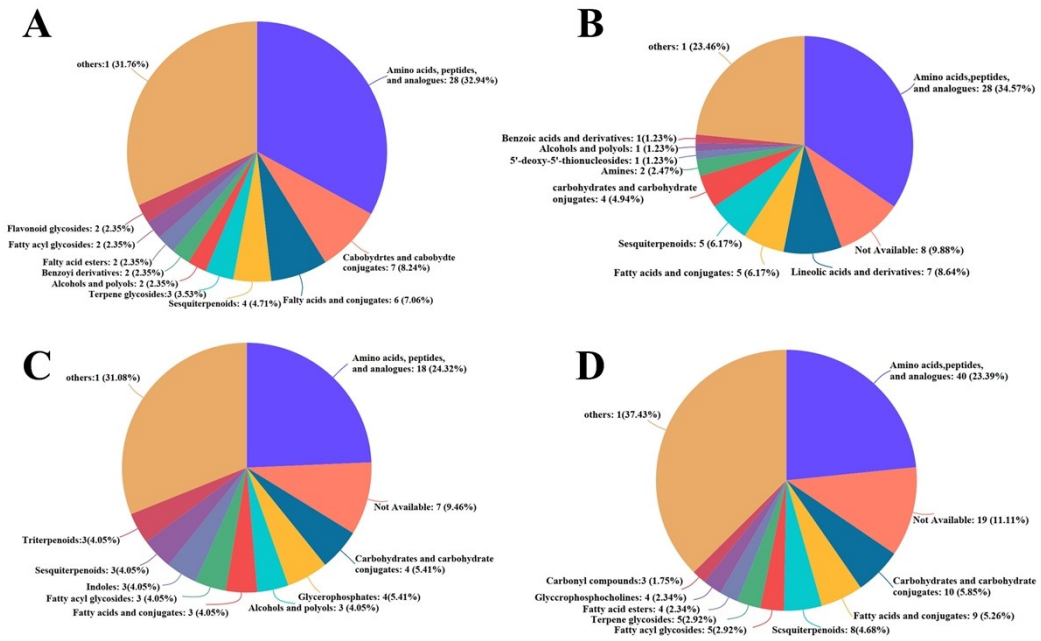

Fig. S11 HMDB compound classification of DMs. (A) CK vs AgNPs. (B) CK vs HemNPs. (C) CK vs MX1. (D) CK vs MX2.

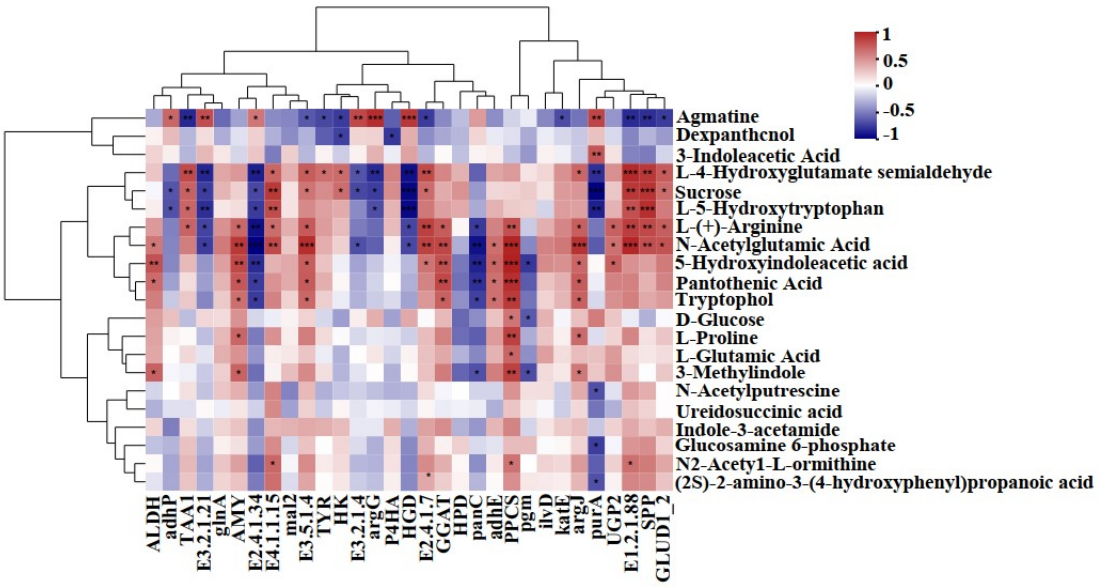

Fig. S12 Heat map of correlation between DEGs and DMs. (* p <0.05, ** p < 0.01, *** p < 0.001)

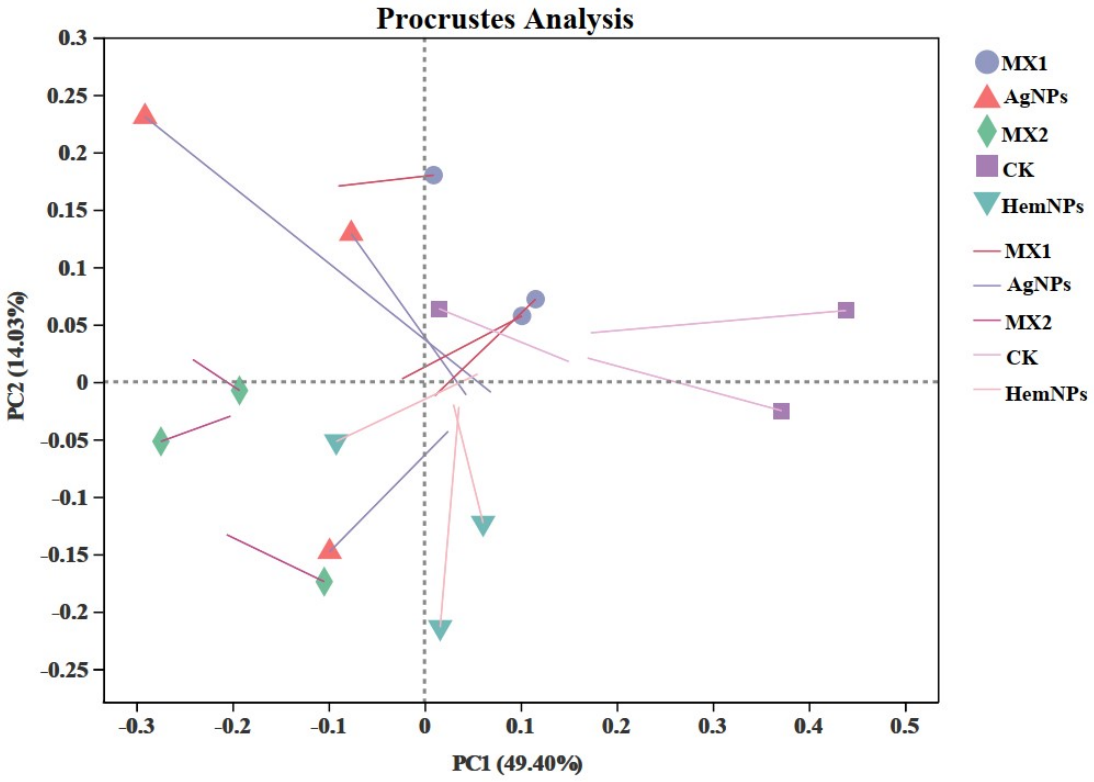

Fig. S13 Procrustes analysis. The short line represents the residual between DEGs expression and metabolite expression.

Table S1 Determination of hydrodynamic diameter of nanoparticles in experimental medium by dynamic light scattering

Test NPs	AgNPs	HemNPs	MX1	MX2
DH ± SD (nm)	212.3 ± 11.6	282.0 ± 10.2	242.6 ± 6.7	387.8 ± 41.8

Table S2 Summary of transcriptome sequencing data and transcriptome assembly

Sample	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
CK1	50287276	7.45E+09	0.0251	97.91	94.08	60.55
CK2	49847954	7.37E+09	0.0251	97.95	94.15	60.49
CK3	48070802	7.12E+09	0.0252	97.87	93.98	60.48
AgNPs1	56884760	8.36E+09	0.0251	97.95	94.15	60.41
AgNPs2	50400780	7.45E+09	0.0248	98.05	94.36	60.55
AgNPs3	50158834	7.44E+09	0.0252	97.87	94.02	60.42
HemNPs1	49963406	7.41E+09	0.0254	97.81	93.86	60.55
HemNPs2	51120380	7.58E+09	0.0251	97.95	94.14	60.52
HemNPs3	44481330	6.59E+09	0.0253	97.87	93.96	60.54
MX1-1	49391708	7.3E+09	0.0252	97.88	94.02	60.41
MX1-2	42479548	6.29E+09	0.025	97.96	94.18	60.43
MX1-3	45511676	6.64E+09	0.025	97.96	94.15	60.32
MX2-1	54428796	7.96E+09	0.0252	97.91	94.03	60.26
MX2-2	51559692	7.49E+09	0.0251	97.91	94.07	59.53
MX2-3	41791392	6.17E+09	0.0252	97.89	94.04	60.35

Table S3 The number of DEGs related to amino acid, carbohydrate, lipid and energy metabolism

	CKvsAgNPs			CKvsHemNPs			CKvsMX1			CKvsMX2		
	Up	Down	Total	Up	Down	Total	Up	Down	Total	Up	Down	Total
Amino acid metabolism	4	0	4	0	2	2	5	6	11	18	31	49
Carbohydrate metabolism	1	2	3	0	8	8	5	8	13	18	44	62
Energy metabolism	3	4	0	0	26	26	3	20	23	24	39	63
Lipid metabolism	0	0	0	0	5	5	1	6	7	7	25	32
Add above	7	6	13	0	38	38	11	34	45	58	111	169